



Rationalisation of the stereochemical outcome of ene-reductase-mediated bio-reduction of α,β -difunctionalised alkenes

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ABSTRACT

The OYE1–3-mediated reductions of some α,β -difunctionalised alkenes, showing on the double bond a nitrile and ester group, are submitted to a careful stereochemical analysis, in order to identify which of the two electron-withdrawing groups (EWGs) is responsible for the activation of the C=C double bond towards reduction and for establishing hydrogen bond interactions within the binding pocket of the enzymes. The results show that for most of these substrates the activating EWG is the CN moiety linked to the prostereogenic olefinic carbon atom. The final stereochemical outcome can be explained through the empirical model which has been recently developed for difunctionalised alkenes activated by carbonyl/carboxyl containing EWGs.

In a single case the activation is due to the COOR group linked to the less substituted olefinic carbon atom: an alternative empirical model is established for this kind of substrates, taking into consideration the OYE-catalysed reductions of β,β' -disubstituted- α -monofunctionalised alkenes.

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1. Introduction

The use of ene-reductases (ERs) for the enantioselective reduction of activated alkenes is currently receiving great interest [1], because of the efficacy shown by this kind of transformation in the synthesis of chiral building blocks for organic chemistry applications [2]. The effects due to the stereochemistry of the starting alkene, to the steric and electronic characteristics of the activating electron-withdrawing groups (EWGs), as well as of other substituents are to be carefully investigated, in order to define the limits and potential of this kind of reaction. The information is essential for including this bio-reduction into the pool of synthetic tools, into which chemists can delve to select the best strategy for the preparation of their target molecules, just as they are accustomed to do when they search among conventional reagents of organic chemistry. The availability of robust enzymatic procedures can facilitate the introduction of biocatalysed steps in modern production processes, bringing along all the advantages of enzymes for sustainability.

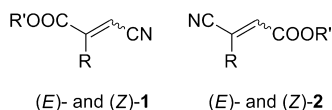
Most of the ERs that have been identified in the last decades belong to the well-known family of Old Yellow Enzymes (OYEs), which are characterised by the presence of a flavin mononucleotide

(FMNH₂) prosthetic group which imparts a yellow colour to purified protein samples. It has been established that the C=C double bond can only be reduced by these enzymes if it is made susceptible to the nucleophilic attack of a hydride (delivered by the reduced flavin mononucleotide prosthetic group) by the presence of an EWG, which is also able to establish hydrogen bonds within the binding pocket of the enzyme [3]. Investigations are to be devoted to define which EWGs can activate alkenes towards OYE-catalysed reductions by themselves and which ones are to be combined with other groups. Up to now, it has been established that α,β -unsaturated aldehydes and ketones, nitroolefins and maleimides are good substrates for this kind of reactions, whereas the OYE-mediated reduction α,β -unsaturated esters is only possible when another EWG is present on the double bond, e.g. a halogen atom linked to the same carbon atom bearing the ester function [4], or an ester [5] or a nitrile [6] group in β position.

We have recently reported [6a] on the reduction of cyano esters (E)- and (Z)-**1** (Scheme 1), precursors of γ^2 -amino acids for foldamer applications, by means of OYE1–3, and we have carried out a detailed analysis of the stereochemical course of the reaction by means of deuterium labelling. Contemporaneously, a paper has been published [6b] describing the reduction of the carbon–carbon double bond of a class of regioisomeric compounds, i.e. (E)- and (Z)-**2** (Scheme 1), by means of isolated OYEs with the aim of providing a biocatalytic route to precursors of GABA analogues, such as pregabalin.

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Scheme 1. Cyano-substituted unsaturated esters subjected to ER-mediated reduction (Ref. [6a] for (E)- and (Z)-1; Ref. [6b] for (E)- and (Z)-2).

On the basis of our experience on substrates **1**, we decided to investigate in more detail the stereochemistry of the bioreduction of compounds **2** and to establish whether the nitrile or the ester function acted as the activating group of the bioreduction, i.e. to determine which of the two EWGs was involved in the formation of hydrogen bonds with the amino acid residues in the active site of OYE1-3.

As a matter of fact, this information is essential to define the binding mode of the substrate, and identify the stereoheterotopic face of the alkene moiety on which the H^+ or H^- is delivered for the formation of the new stereogenic centre. Under the practical point of view, it is useful to identify the structural requisites influencing this arrangement, in order to achieve the control on the final stereochemical outcome of the reaction through a guided substrate-engineering strategy.

The results obtained from this study have been also used to better define the empirical model we have recently developed [6a] to rationalise the experimental data on OYE1-3 mediated reductions of activated alkenes, and to describe the structural prerequisites for the optimal arrangement of the substrates within the binding site of the enzymes. A distinction could be made between trisubstituted alkenes for which the activating EWG was linked to the prostereogenic carbon atom, and those activated by the other functional group.

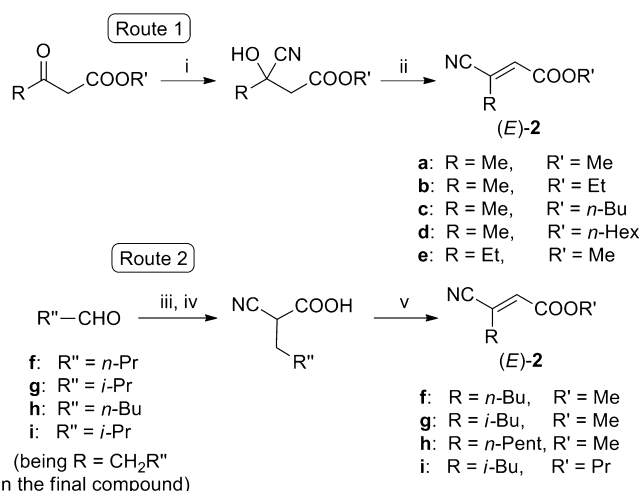
2. Experimental

2.1. General procedure for OYE1-3 mediated biotransformations of substrates (E)-2a-i (screening)

A solution of the substrate in DMSO (10 μ L, 500 mM) was added to a potassium phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μ mol), NADP⁺ (0.1 μ mol), GDH (4 U) and the required OYE (0.89 μ M). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (2 $\times$ 250 μ L), centrifuging after each extraction (15,000 $\times$ g, 1.5 min), and the combined organic solutions were dried over anhydrous Na₂SO₄. Two replicates were performed for each biotransformation: no significant differences were observed for conversion and enantiomeric excess values.

2.2. General procedure for OYE1-3 mediated biotransformations (50 mg scale)

For substrates (E)-2a-h a similar protocol was followed on a larger scale, employing the OYE which provided the best conversion and/or ee, in order to isolate and characterise the corresponding reduced product. A solution of the suitable cyano ester in *i*-PrOH (1 mL, 250 mM) was added to a potassium phosphate buffer solution (25 mL, 50 mM, pH 7.0) containing the required OYE (0.89–2.66 μ M), GDH (100 U), glucose (1 mmol, 180 mg) and NADP⁺ (5 μ mol, 3.7 mg). The reaction was monitored by GC until complete conversion. The mixture was then extracted with EtOAc (3 $\times$ 10 mL) and submitted to column chromatography (*n*-hexane with increasing amount of EtOAc), when conversion was not complete in order to purify the reduced product from the starting material.



Scheme 2. Synthesis of cyanoesters (E)-2. Reagents and conditions: (i) Me₃SiCN, DMSO–water 5:1; (ii) SOCl₂, Py, 100 °C; (iii) cyanoacetic acid, cat. NH₄OAc in toluene; (iv) NaBH₄ in water and NaHCO₃; and (v) CHOCOR', Ac₂O, Py.

2.3. Bioreduction procedure for the preparation of monodeuterated samples

A solution of the substrate in *i*-PrOH (100 μ L, 500 mM) was added to potassium phosphate buffer solution (5.0 mL, 50 mM in D₂O, pH 7.0) containing glucose (20 μ mol), NADH (75 μ mol) and the required OYE (1.11 μ M). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (3 $\times$ 5.0 mL), centrifuging after each extraction (3000 $\times$ g, 1.5 min), and the combined organic solutions were dried over anhydrous Na₂SO₄.

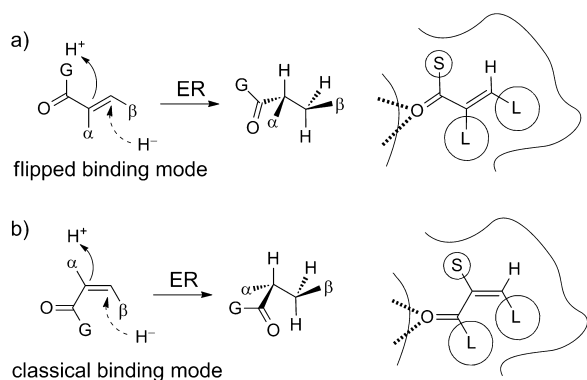
3. Results and discussion

The known data [6b] of the bioreductions of compounds **2** can be summarised as follows. The best values of conversion and enantioselectivity were achieved by using OYE1-3. (E)-stereoisomers were generally converted into (S)-reduction products with high enantioselectivity, whereas the conversion of (Z)-cyano esters into the corresponding (R)-enantiomers decreased with the increasing steric demand of the substrates. Reducing the size of the ester moiety from ethyl to methyl had a strong positive impact on conversions, thus suggesting activation due to the ester group. An interesting case of exceptional behaviour of OYE3 was observed as for the enantioselectivity of the reduction of the cyano ester precursor of pregabalin.

We decided to start a systematic investigation of the stereochemical course of these reactions, by preparing other substrates of type **2**, showing (E) stereochemistry at the double bond and increasing steric hindrance either at the ester moiety (**2a–d**) or at the prostereogenic olefinic carbon atom (**2e–h**). Compound **2i** was also prepared to investigate the peculiar enantioselectivity of OYE-mediated reductions of isobutyl substituted cyanoesters.

A different synthetic approach was chosen with respect to that described in ref. 6b, in order to optimise the preparation of (E)-stereoisomers of cyano esters **2**. Derivatives (E)-2a–e were synthesised starting from the corresponding β -keto ester according to route 1 (Scheme 2) which afforded only the desired configuration of the C=C double bond. Route 2 was employed to obtain compounds (E)-2f–i, which were recovered from the reaction mixture and separated from the (Z)-stereoisomer by column chromatography.

The absolute configuration of the reduced products was determined by conversion either in the dimethyl ester or in the diacid derivatives (by treatment with refluxing methanol and a catalytic

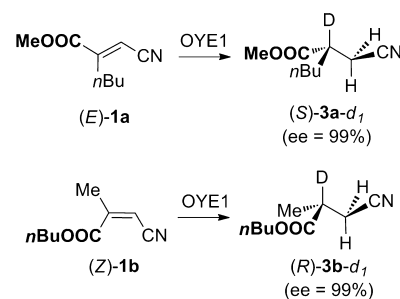


Scheme 3. Empirical model for the ER-mediated reductions of olefins activated by carbonyl/carboxyl moieties linked to the prostereogenic carbon atom (G is the substituent linked to the C=O moiety of the EWG; α and β are the substituents in alpha and beta position with respect to the EWG; S and L stand for small and large, respectively).

amount of sulphuric acid, or by reaction with refluxing concentrated hydrochloric acid, respectively), or by comparison with known specific optical rotation data.

The results of OYE 1–3 biotransformations are reported in Table 1. The analysis of these data showed that a decrease in enantioselectivity readily occurred when the steric hindrance of the alkyl substituent linked to the stereogenic carbon atom increased, except for compound (E)-2i. A decrease of conversion values was observed only in the reduction of the branched derivatives (E)-2g and i, probably as a consequence of a reduced reaction rate. As for substrates 2a–d, characterised by a methyl group on the more substituted olefinic carbon atom, the increasing length of the alkoxy chain perturbed neither the enantiomeric excess, nor the conversion values.

We considered these data with regard to the stereochemical analysis we had previously performed on the bioreduction of regioisomeric cyano esters 1. We were also interested in the investigation of the reactions of substrate (E)-2g, for which a change in enantiopreference had been already observed [6b] by comparing the results obtained with OYE 1 and 2 with those collected with OYE3. For this reason we prepared also the corresponding propyl ester 2i which was described to afford invariably the (S)-enantiomer by OYE1–3 mediated reductions. If the activating EWG, i.e. the one which is involved in the formation of hydrogen bonds with His191 and/or Asn194 in the active site of OYE1–3 (numbering according to PDB structure code 1OYB), contains a C=O group and it is linked to the prostereogenic olefinic carbon atom, two binding modes have been described in the literature till now: a “classical” and a “flipped” one (Scheme 3) [3c,7]. Recently [8], a modification of the arrangement of the substrate in the enzyme active site induced by rotation of the alkenyl fragment on the C α –CO single bond has been hypothesised. This conformational change produces a different orientation of the substrate in the so-called “flipped” binding mode: the stereoheterotopic faces exposed to H $^+$ and H $^-$ addition are indeed the same. Our past experience on regioisomeric cyano esters 1 had allowed us to draw the conclusions which are depicted in Scheme 3 for trisubstituted alkenes, activated by an EWG containing a carbonyl/carboxyl moiety. In the drawing G represents the group linked to the C=O moiety of the EWG, and α and β are the substituents of the double bond in alpha and beta position with respect to the EWG. The so-called “flipped” binding mode (Scheme 3a) is preferred when G is small (S) and α is large (L). The favoured stereochemistry of the double bond is the one shown in the picture with the large substituent at C β (L) on the opposite side of the activating group, i.e. (E) stereochemistry for most substrates.



Scheme 4. Results of OYE1-mediated biotransformations of derivatives (E)-1a and (Z)-1b.

Conversely, when G is large and α is small, the “classical” binding mode (Scheme 3b) is preferentially adopted. The optimal stereochemistry of the double bond is the one depicted in Scheme 3b with the large substituent at C β (L) on the same side of the EWG, i.e. (Z) stereochemistry for most substrates.

For example, for substrates (E)-1a and (Z)-1b (Scheme 4) the activating group was identified to be the ester moiety by deuteration experiments: as a matter of fact, the use of D₂O as a solvent of the OYE-mediated reduction, in the presence of a stoichiometric quantity of NADH is known [9] to promote the incorporation of a deuterium atom at C α with respect to the activating group. This information, combined with the absolute configuration of the final products, i.e. (S)-3a and (R)-3b respectively, allowed then the binding mode to be defined: a flipped arrangement for (E)-1a and a classical one for (Z)-1b. In order to give the most consistent representation of the active site, we adopted the convention of drawing the substrates in such a way that the activating EWG is always shown on the left and highlighted in boldface character. According to this representation, the hydride is always delivered from below the plane, while the proton (or in this particular case the D $^+$) is captured from above.

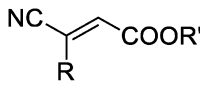
The bioreduction of the corresponding stereoisomers, (Z)-1a and (E)-1b, occurred with lower conversions and enantioselectivities, because the stereochemistry of the double bond was not the optimal one for the binding mode induced by the steric hindrance of G and α .

In order to make analogous considerations on the most reactive binding mode of substrates 2a–i it was necessary to establish whether the activating functional group was the nitrile or the ester moiety. With this aim, derivatives (E)-2b, g and i were submitted to deuteration experiments: the analysis of the ^1H NMR spectra of the products (Fig. 1b, and S1b and S2b in the Supplementary Data) showed that in the OYE2-mediated reduction of these substrates, affording the (S) enantiomer of the corresponding monodeuterated reduced products 4b, g, and i, the activating group was the CN moiety linked to the most substituted olefinic carbon atom (Scheme 5).

Unexpectedly, the nitrile function resulted to be involved in the hydrogen bond interaction within the enzyme active site. The binding of the CN group to the amino acid residues is preferred to the coordination of the carboxylic moiety, in spite of the peculiar linear geometry of the carbon nitrogen triple bond.

The substrate binding modes established for the OYE2-mediated reduction of compounds of (E)-2b, g, and i are in accordance with the empirical model we have proposed (Scheme 3): the activating CN moiety has no G group and the steric hindrance of the α substituent determines the arrangement. Thus, a flipped binding mode results to be the most reactive one and the (E)-stereochemistry of the substrate is the most suitable one for this binding mode.

Table 1
Results of OYE1-3-mediated reductions of substrates (*E*)-**2a–i**.

		Substrate	OYE 1		OYE2		OYE3	
R	COOR'		c ^a (%)	ee ^b (%)	c ^a (%)	ee ^b (%)	c ^a (%)	ee ^b (%)
Me	COOMe	(<i>E</i>)- 2a	99	99 (S)	99	99 (S)	99	99 (S)
Me	COOEt	(<i>E</i>)- 2b	99	99 (S)	99	99 (S)	99	99 (S)
Me	COO <i>n</i> Bu	(<i>E</i>)- 2c	99	99 (S)	99	99 (S)	99	99 (S)
Me	COO <i>n</i> Hex	(<i>E</i>)- 2d	84	99 (S)	98	99 (S)	99	99 (S)
Et	COOMe	(<i>E</i>)- 2e	99	99 (S)	99	99 (S)	91	66 (S)
<i>n</i> -Bu	COOMe	(<i>E</i>)- 2f	99	60 (S)	99	70 (S)	92	20 (S)
<i>i</i> -Bu	COOMe	(<i>E</i>)- 2g	46	50 (S)	53	76 (S)	60	88 (R)
<i>n</i> -Pent	COOMe	(<i>E</i>)- 2h	94	44 (S)	78	69 (S)	91	rac
<i>i</i> -Bu	COO <i>n</i> Pr	(<i>E</i>)- 2i	56	99 (S)	57	99 (S)	27	99 (S)

^a c = conversion percentage, calculated by GC analysis of the crude mixture after 24 h reaction time (these values are not significantly different from percentage yields, as neither degradation nor side-product formation was observed; isolation yields are reported in the Supplementary Data).

^b Calculated by GC analysis on a chiral stationary phase.

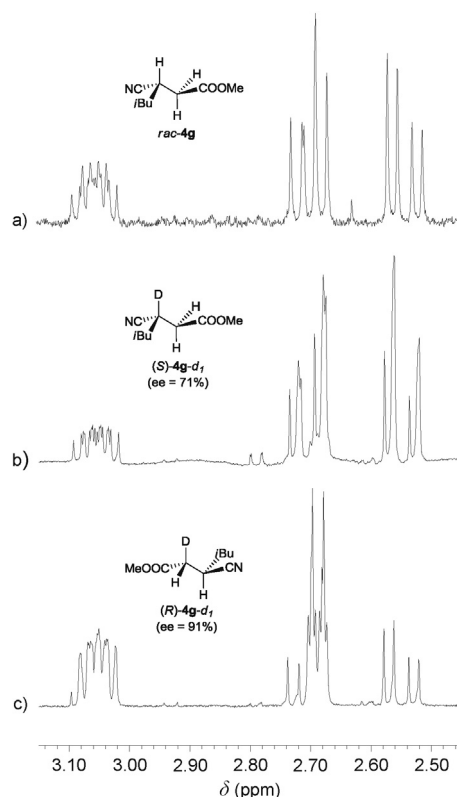
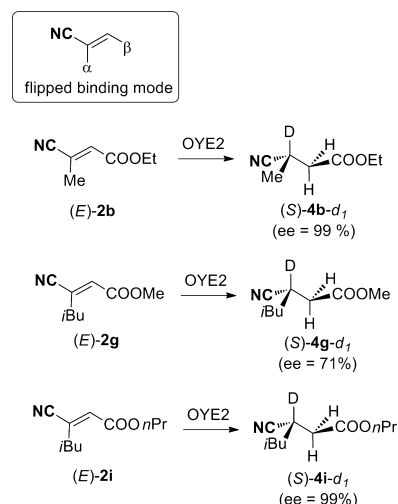


Fig. 1. (a) ¹H NMR spectrum of *rac*-**4g**; (b) ¹H NMR spectrum of (*S*)-**4g**-*d*₁ obtained from (*E*)-**2g** by OYE2-mediated reduction in D₂O in the presence of stoichiometric NADH (c = 99%, ee = 71%); (c) ¹H NMR spectrum of (*R*)-**4g**-*d*₁ obtained from (*E*)-**2g** by OYE3-mediated reduction in D₂O in the presence of stoichiometric NADH (c = 90%, ee = 91%).

Within this class of compounds, the increase of the bulkiness of the α group induces a decrease in enantioselectivity, that had not been observed for derivatives **1**: e.g. (*E*)-**1a** was converted by OYE 1–3 into (*S*)-**3a** with ee = 99%, whereas (*E*)-**2f** was converted into poorly enriched (ee = 20–70%) compounds (*S*)-**4f**. This decrease in enantioselectivity reflects a less definite preference for a certain binding mode and the possibility of achieving more than one arrangement within the binding pocket of the enzyme, affording opposite enantiomers upon reduction.

An interesting behaviour was shown by the two isobutyl derivatives (*E*)-**2g** and **i**. Compound **2g** was converted into (*S*)-**4g** in modest conversion yields (c = 46–53%) and ee values (ee = 50–76%) by OYE1 and 2, whereas the OYE3-mediated reaction afforded the



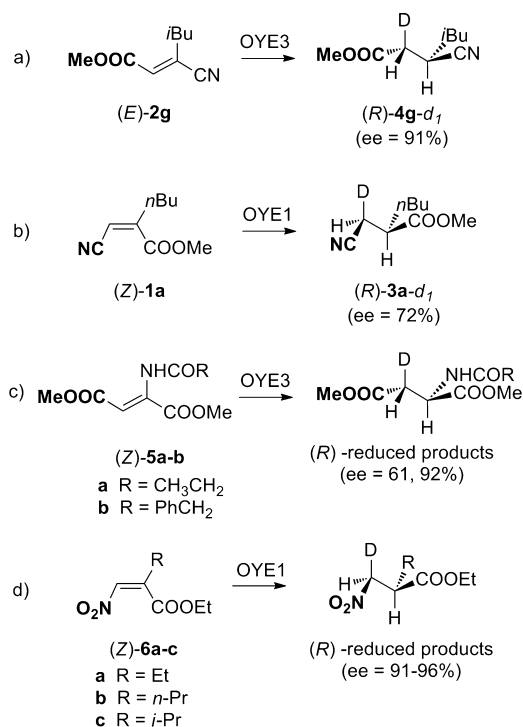
Scheme 5. Results of deuteration experiments on substrates (*E*)-**2b**, **g** and **i** performed in the presence of OYE2.

(*R*)-**4g** with c = 60% and ee = 88%. Deuteration experiments (c = 90% and ee = 91%) highlighted that in this latter reaction the activating group was no longer the nitrile, but the ester function (Fig. 1c and Scheme 6a).

In the series of cyanoesters of type **1** we had observed an exchange of the activating EWG between the ester and the nitrile moieties in the OYE1-mediated reduction of substrate (*Z*)-**1a**, to afford (*R*)-**3a** with ee = 72% (Scheme 6b). To our knowledge, in the literature there are only two more examples of α,β-difunctionalised alkenes for which it was experimentally demonstrated that the activating EWG was the one on the less substituted olefinic carbon atom: the two amido fumarates (*Z*)-**5a** and **b** in OYE3 reductions [9] (Scheme 6c), and the nitroacrylates (*Z*)-**6a–c** treated with OYE1 [10] (Scheme 6d).

In all these cases the substituent α linked to the carbon atom bearing the activating EWG is only a hydrogen atom, and the steric hindrance of the two groups at β position controls the arrangement of the substrate in the binding pocket. A flipped binding mode is assumed by alkenes (*E*)-**2g** and (*Z*)-**5a,b** in the active site of OYE3, and a classical arrangement is adopted by (*Z*)-**1a** and (*Z*)-**6a–c** in the binding pocket of OYE1.

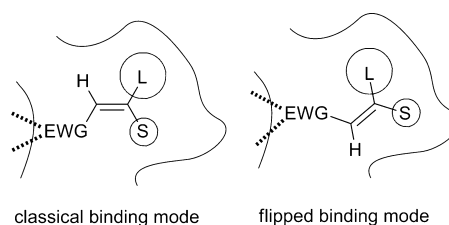
We tried to rationalise these results by taking also into account the literature data for OYE1–3 mediated reductions of trisubstituted monofunctionalised alkenes showing no substituents at the carbon atom linked to the activating EWG. The available data are shown



Scheme 6. Difunctionalised alkenes activated by the EWG on the less substituted carbon atom (this work, Refs. [6a,9,10]).

in [Scheme 7](#), some of which have been collected employing whole cells of baker's yeast (BY, *Saccharomyces cerevisiae*, which expresses OYE2 and OYE3) rather than isolated ERs. BY and OYE1–3-mediated reductions of (*E*)-1-nitro-3-aryl-1-propene derivatives afforded the (*R*)-enantiomer of the reduced products [11], and BY converted (*E*)-3-arylbut-2-enals into the corresponding (*S*)-saturated primary alcohols [12].

For these (*E*)-substrates the only activating EWG (NO₂ or CHO) is linked to less substituted olefinic carbon atom, and the reactions occur through the same preferred classical binding mode, in



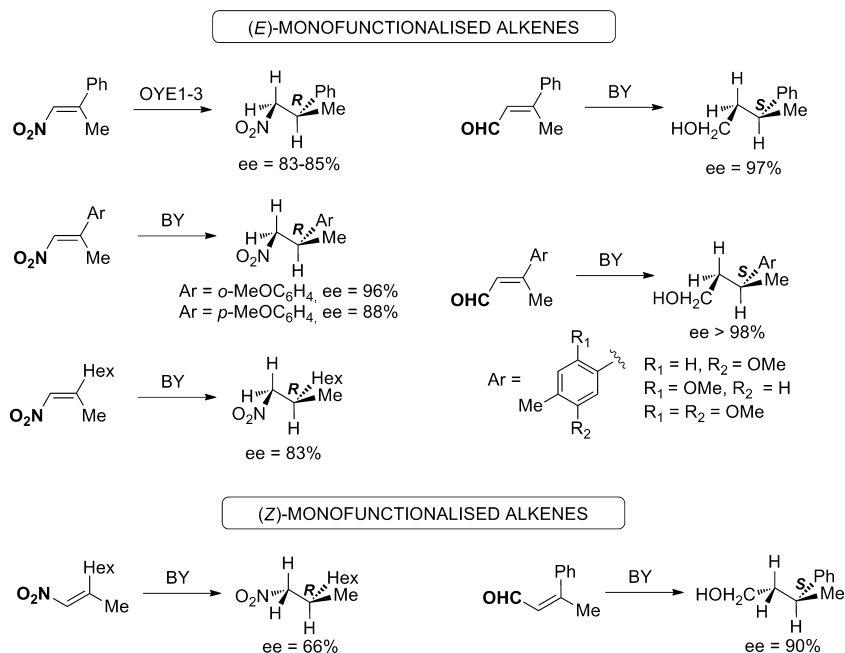
Scheme 8. Empirical model for the ER-mediated reductions of olefins activated by an EWG linked to the less substituted olefinic carbon atom.

which the largest group at the prostereogenic carbon atom is on the opposite side with respect to the EWG ([Scheme 7](#)).

The position of the most hindered group at the β carbon atom is maintained also in the arrangement of the (*Z*)-stereoisomers, as it has been shown for (*Z*)-β-methylcinnamaldehyde and for (*Z*)-2-methyl-1-nitrooct-1-ene, causing a flipped arrangement to be adopted ([Scheme 8](#)), and the same absolute configuration of the resulting reduced product to be obtained ([Scheme 7](#)). However, the optimal stereochemistry of the double bond, affording the highest values of enantioselectivity, seems to be the one in which the largest group in β position is on the opposite side to the activating EWG.

In the case of substrate (*E*)-**2i**, OYE1–3-mediated reductions gave the (*S*)-reduced product in modest yields and very high enantioselectivity (ee = 99%). The deuteration experiments were repeated also with OYE3 and highlighted that the CN moiety was still the activating group. The distribution of substituents on the double bond has here two effects: (i) it limits the conformational freedom of the substrate, and (ii) it is however suitable for finding a preferred arrangement. Enantioselectivity values are related to the preference for a particular enzyme–substrate complex. In the case of compound **2i** the steric hindrance of substituents makes the formation of the complex a more energy demanding step, but it also limits the possible arrangement.

As for derivatives **2a–d**, their reduction data are in agreement with the fact that being the nitrile the activating group, no effect is caused by the increasing steric hindrance at the ester moiety.



Scheme 7. Literature data of OYE1–3-mediated reductions of β,β'-disubstituted-α-monofunctionalised alkenes (Ref. [11,12]).

4. Conclusions

The experimental data show that trisubstituted α,β -difunctionalised alkenes, such as cyano esters **2**, and, as we have previously shown, cyano esters **1** [6a] and diesters [5], have a preference for binding in the enzyme active site through the EWG linked to the prostereogenic olefinic carbon atom. The most reactive binding mode does not seem to be influenced by the electronic and geometric characteristics of the activating functional group: no preference is shown for trigonal functionalities, and even the linear CN can afford a suitable hydrogen bond interaction within the enzyme active site.

All the information so far collected on the factors controlling the stereochemical outcome of OYE1-3-mediated reactions has been summarised in figure S3 (see Supplementary Data). When the activating group contains a carbonyl/carboxyl moiety and it is linked to the prostereogenic carbon atom, the steric hindrance of the α and G fragments control the arrangement of the substrate. A flipped or classical arrangement is adopted according to the relative dimensions of the two groups: bulky G groups promote a switching of the binding mode and a subsequent change of the absolute configuration of the stereogenic centre. The two binding modes, defined according to the spatial position of the activating EWG which establishes on which stereoheterotopic face of the alkene the H^+ addition occurs, are characterised by a preference for a certain stereochemistry of the C=C double bond. This empirical model (Figure S3) can be extended also to alkenes for which the activating EWG is a CN moiety with no G group.

If the activating group is linked to the less substituted carbon atom, then α is a hydrogen atom, and the absolute configuration of the new stereogenic centre is controlled by the structural requisite of positioning the most sterically demanding group at C_β in a well-established spatial arrangement (Figure S3). According to this condition, (*E*) and (*Z*) stereoisomers afford the same enantiomer by adopting a classical and flipped binding mode, respectively, with the (*E*)-substrates usually giving the highest enantioselectivity values.

In the case of difunctionalised alkenes only a few examples of switching of the activating EWG was observed, thus preventing from establishing the structural requisites promoting this exchange, which causes a variation of enantioselectivity. When the activating EWG is on the less substituted olefinic carbon atom, the empirical model for monofunctionalised alkenes can be employed to rationalise the stereochemical outcome. The condition is to accommodate the largest group at C_β in a specific spatial region of the pocket, thus resulting in a classical or flipped binding mode according to the stereochemistry of the double bond.

All the information herein collected can help in defining new potential substrates for the optimal enantioselective reduction of the double bond, in the aim of obtaining a control on enantioselectivity and conversion through a wise structural definition of the substrate, as a complementary approach to the optimisation of new stereodivergent ERs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molcatb.2013.12.020>.

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